

# **Nonlinear optics as a detection scheme for biomimetic sensors: *SFG spectroscopy of hybrid bilayer membrane formation***

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## **ABSTRACT**

Vibrational spectra of biomimetic membranes have been obtained using a broad-band approach to sum frequency generation (SFG). A new innovation, broad band SFG (BBSFG) allows for high quality SFG spectra with rapid collection times. With the BBSFG approach, we have followed *in situ* the formation of a hybrid bilayer membrane (HBM) from the reorganization of phospholipid vesicles at alkanethiol monolayers.

**Keywords:** nonlinear optics, sum frequency generation, lipid bilayers, self-assembled monolayers, vibrational spectroscopy

## **1. INTRODUCTION**

Biological membranes contain much of the molecular machinery of cellular recognition and sensing. With this insight, biosensors and biocompatible materials could be designed from components of biological membranes and from intact cell membranes. Our group has developed a biomimetic material, the hybrid bilayer membrane, which in its simplest form consists of a phospholipid monolayer on top of an alkanethiol self-assembled monolayer (SAM) on gold.<sup>1-3</sup> Hybrid bilayer membranes (HBM) have been shown to have many properties of cell and model membranes, but with added ruggedness and extended stability. Additionally, HBMs are electrically-addressable using the underlying gold film as a working electrode; electrochemical measurements have been used to study the interactions of adsorbates with HBMs.<sup>2,3</sup>

A major advantage of the HBM is the flexibility and ease of its preparation. One approach to HBM fabrication is to expose the hydrophobic alkanethiol surface to a solution of phospholipid vesicles which leads to vesicle fusion.<sup>2</sup> After the incubation, the unfused vesicles are removed by rinsing. Figure 1 gives a schematic of the dominant mechanism of vesicle fusion that leads to HBM formation.<sup>4</sup> The kinetics of vesicle fusion in HBM formation has been studied by surface plasmon resonance, but to date no spectroscopic information has been obtained to verify the proposed mechanism. Since the mechanism of bilayer formation may affect the resulting composition of the HBM, a spectroscopic method of following HBM formation and structure is highly desired. Furthermore, properties likely to be important to the functioning of an HBM-based biosensor, such as the phospholipid gel-to-liquid crystalline phase transition temperatures, have yet to be determined due to our lack of an *in situ* spectroscopic probe. \*

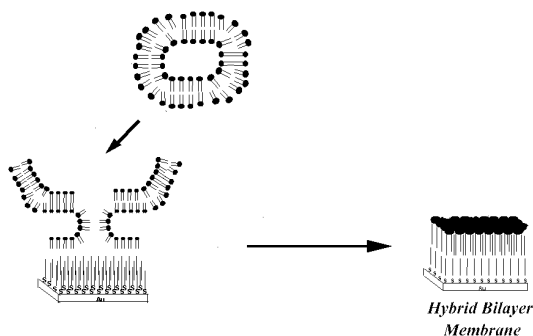


Figure 1: The proposed mechanism of vesicle fusion to form a phospholipid/alkanethiol HBM.

Sum frequency generation (SFG), a nonlinear optical vibrational spectroscopic technique, is among several methods we are developing as *in situ* probes of HBMs. Historically used for study of interfaces<sup>5-8</sup>, SFG has only recently been applied to the surface studies of biological molecules.<sup>9,10</sup> SFG offers powerful advantages due to its high surface selectivity, submonolayer sensitivity, and excellent spatial, spectral and temporal resolution.<sup>6</sup> SFG is capable of *in situ* measurements at biological interfaces and potentially able to give direct information about the structure, orientation, aggregation, and organization of surface-associated biomolecules.

In SFG, two laser beams of different wavelengths, one visible (V) and one infrared (IR), are simultaneously reflected from an interface. Due to the nonlinear properties of the surface, a visible photon  $\omega_v$  and an infrared photon  $\omega_R$  can interact to create a third photon with a frequency that is at the sum of the two photon frequencies,  $\omega_{SF} = \omega_v + \omega_R$ . This occurs through a nonlinear polarization  $\mathbf{P}^{(2)}$  of the surface caused by the electric fields ( $\mathbf{E}$ ) of the incident light and the second order nonlinear susceptibility  $\chi^{(2)}$  of the surface.<sup>5,8</sup>

$$\mathbf{P}^{(2)}(\omega_{SF}) = \chi^{(2)} : \mathbf{E}(\omega_v) : \mathbf{E}(\omega_R). \quad (1)$$

The symmetry properties of  $\chi^{(2)}$  are the origin of the exceptional surface specificity of SFG.<sup>5-7</sup> Within the electric dipole approximation,  $\chi^{(2)}$  is zero for centrosymmetric media, i.e., achiral solutions, such as air, water, buffers, etc. An interface between two media, such as gas/solid or liquid/solid, naturally breaks centrosymmetry which allows  $\chi^{(2)}$  to be nonzero, and SFG may occur. Therefore, SFG signals are only generated by molecules at the interface and have zero background from molecules in solution.

SFG activity requires noncentrosymmetric vibrations, or noncentrosymmetric arrangements of vibrations, which leads to the requirement of an simultaneous nonzero IR dipole and a nonzero Raman transition dipole for SFG activity.<sup>5</sup> The resultant SFG spectra can therefore be less complex than IR or Raman spectra and, in principle, yield more information about molecular orientation. Furthermore, since the relationship between SFG activity and symmetry is closely tied to molecular structure, both the presence and absence of vibrational bands in SFG spectra can be helpful in interpreting complex surface IR and Raman spectra.

SFG is sensitive to the orientation (up/down, tilt angle) of interfacial molecules.<sup>5</sup> Due to the asymmetric nature of an interface, amphiphilic molecules often adsorb with a net orientation in the direction normal to the interface. As  $\chi^{(2)}$  is a third rank tensor, the sign of  $\chi^{(2)}$  inverts with a 180 degree rotation of molecular orientation, and thus the sign of a SFG band changes. Consequently, the net orientation of an adsorbate can often be inferred from the observed sign of the SFG bands. Additionally, a layer of adsorbates with random orientations (in the direction normal to the surface) will mutually cancel and yield no net SFG bands.

For surface coverages up to a monolayer,  $\chi^{(2)}$  can be modeled as:<sup>5</sup>

$$\chi^{(2)} = N_s \langle \beta \rangle \quad (2)$$

where  $N_s$  is the number density of molecules on the surface and  $\beta$  is the second-order optical polarizability of an individual molecule. The brackets indicate an average over all orientations of the molecules. The intensity of the SFG bands is proportional to the square of  $\chi^{(2)}$ . Therefore, SFG band intensities can be used to follow the adsorption of molecules at a surface, for example, the adsorption of phospholipids during the formation of an HBM.

## 2. EXPERIMENTAL<sup>11</sup>

Perdeuterated octadecanethiol (d-ODT) was synthesized and purified by Dave Vanderah, NIST. Head group-deuterated dipalmitoyl phosphatidylcholine (d-13 DPPC) was obtained from Avanti Polar Lipids. Figure 2 shows the molecular structure of d-13 DPPC. Deuterium oxide (99.9 %) was obtained from Cambridge Isotopes.

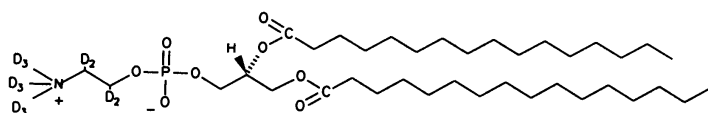


Figure 2: (a) d13-DPPC molecular structure

Glass microaqueduct (MA) slides (Bioprotechs) were the starting substrates for the sample preparations. The MA slides were cleaned using a potassium persulfate/sulfuric acid mixture, rinsed in high purity water, and dried using acetone and filtered nitrogen. A 0.4 nm chromium adhesion layer followed by a 200 nm gold layer was sputter deposited onto the MA slides. The coated substrates were incubated overnight in 1 mM d-ODT solutions in 200 proof ethanol (99.5 %, Aldrich ) to form hydrophobic SAMS. Following incubation, the substrates were rinsed and sonicated in ethanol.

Phosphate buffered saline (PBS)(20 mM potassium phosphate, 150 mM potassium chloride, pH 7.20) in D<sub>2</sub>O was used as the solvent for HBM formation and rinsing. Phospholipid vesicles were prepared using an injection method that has been shown to produce uniform ~ 0.01  $\mu$ m diameter unilamellar vesicles.<sup>2</sup> To prepare vesicles, 2  $\mu$ moles of phospholipid in chloroform was dried under nitrogen and held overnight under vacuum to remove all solvent. The phospholipid was redissolved in 50  $\mu$ L of dry isopropanol and injected with a Hamilton syringe into 1 mL of vortexing PBS. This produced 2 mM vesicles that were subsequently diluted to 0.2 mM before use.

The laser system and experimental approach used in our broad band SFG (BBSFG) measurements has been described in detail elsewhere.<sup>12</sup> Briefly, a nominally 100 fs laser system generates broad bandwidth (BB) IR pulses that are mixed with narrow bandwidth visible pulses(800 nm) at the interface of interest. The SF light at nominally 645 nm is collected and dispersed in a spectrograph. A scientific grade CCD detector is used to detect the entire spectrum of the SF light (e.g., 600  $\text{cm}^{-1}$ ) in parallel. The instrumental spectral resolution is determined by the spectrometer and the bandwidth of the nonresonant visible pulse. Experiments reported herein use BB IR pulses in the frequency range of 2700-3100  $\text{cm}^{-1}$ . The IR wavelength is calibrated by inserting a gas cell containing HCL in the IR beam before upconversion, which imprints a HCL absorption spectrum on the SFG spectrum. All SFG spectra have been smoothed using a bionomial average.

The sample cell is a model FCS2 microscopy chamber from Bioprotechs (Butler, PA). Within the chamber, a 25 micron Teflon spacer is sandwiched between a 2 mm calcium fluoride entrance window and the SAM surface on the gold-coated

microaqueduct slide. The input and output ports on the chamber align with the microaqueducts to allow lamellar flow across the sample. A peristaltic pump is used to pull solution through the cell at a flow rate of 0.2 mL/min. The surface was kept in uninterrupted contact with flowing PBS/D<sub>2</sub>O before, throughout, and after collection of SFG spectra. A 15 mL centrifuge tube was used as a sample reservoir and Tygon tubing (0.16 mm ID, 0.8 mm OD) connected the reservoir and the pump to the sample cell. The cell is mounted vertically, perpendicular to the optical table. The IR and visible beams are incident on the calcium fluoride window at 70 degrees in a nearly collinear geometry. Refraction gives an incident angle of nominally 45 degrees at the buffer/SAM interface.

### 3. RESULTS & DISCUSSION

Figure 3 shows SFG spectra before and throughout d13-DPPC vesicle fusion at the buffer/d-ODT SAM interface. The spectra in Figure 3 were each ratioed to the SFG spectrum of the buffer/d-ODT interface to remove the nonresonant background of the underlying gold film. The spectra were each collected over 60 seconds at a resolution of 4 -5 cm<sup>-1</sup>. The input beams were p-polarized and the SFG wavelength was centered at 643 nm.

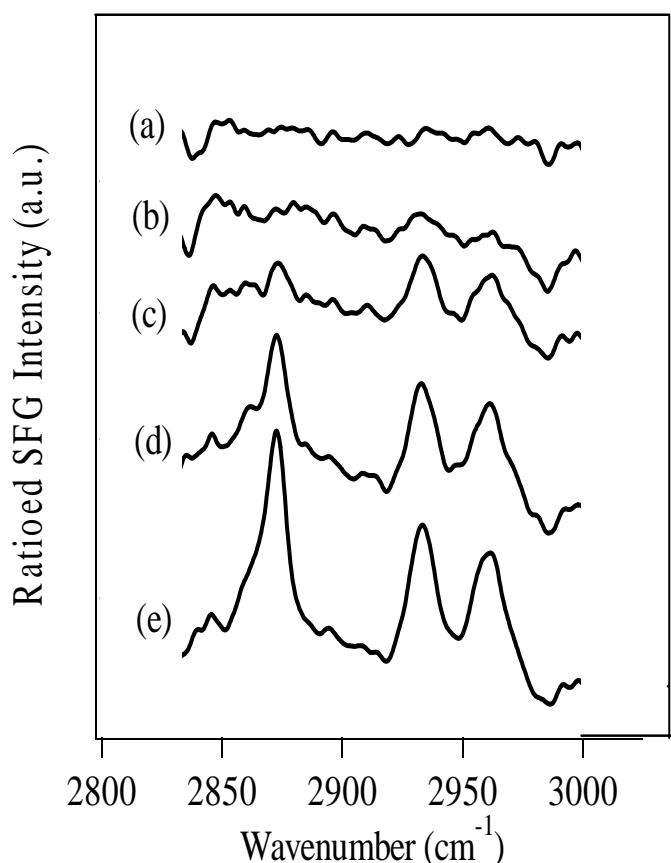


Figure 3: SFG spectra before and throughout d13-DPPC vesicle fusion at the buffer/d-ODT SAM interface. The spectra were collected at (a) 2 minutes, (b) 6 minutes, (c) 12 minutes, (d) 24 minutes, and (e) 46 minutes after the addition of vesicles to the sample reservoir.

The series of spectra in Figure 3 show the growth of vibrational bands over time. d-13 DPPC is fully deuterated in its head group and has hydrogenated methylene and methyl groups only within its acyl backbone and chains. The strong features in the spectra in Figure 3 are assigned to methyl vibrational modes: the CH<sub>3</sub> symmetric stretch at 2873 cm<sup>-1</sup>; the CH<sub>3</sub>

asymmetric stretch at  $2961\text{ cm}^{-1}$ ; and a Fermi resonance between the  $\text{CH}_3$  symmetric stretch and a  $\text{CH}_3$  bend overtone at  $2934\text{ cm}^{-1}$ .<sup>5</sup> Methylene resonances would be expected at  $2850\text{ cm}^{-1}$ , assigned to the  $\text{CH}_2$  symmetric stretch, and a broad band in the region of  $2890 - 2930\text{ cm}^{-1}$ , assigned to a Fermi resonance between the  $\text{CH}_2$  symmetric and the  $\text{CH}_2$  bend overtones, and the  $\text{CH}_2$  asymmetric stretch. The observation of no or very weak methylene resonances indicates that the acyl chains of d-13 DPPC are highly ordered, and in predominantly an *all-trans* configuration, as  $\text{CH}_2$  modes of *all-trans* acyl chains are symmetry forbidden in SFG.<sup>5</sup>

For monolayers on metal surfaces, SFG vibrational bands can be positive, as in Figure 3, or negative depending on experimental factors such as optical geometry or monolayer properties (e.g, polar orientation about the surface normal).<sup>5</sup> For our experimental approach, we have established the relationship between the sign of the SFG bands and a monolayer's molecular orientation using well-characterized systems including octadecanethiol SAMs on gold and dodecanol on d-ODT SAMs on gold. Negative bands correspond to methyl groups pointing away from the surface and positive bands correspond to methyl groups pointing toward the surface. Using this relationship, the positive bands in our SFG spectra of adsorbed d-13 DPPC indicate that the terminal methyl groups have a net polar orientation towards the d-ODT monolayer. This is consistent with the expected orientation of the phospholipid acyl chains due to Van der Waals' interactions with the d-ODT alkane chains.<sup>4</sup>

Figure 4 shows the effect of rinsing on the adsorbed d13-DPPC. The spectrum in (a) is of the adsorbed d-13 DPPC in contact with the vesicle solution; the spectrum in (b) is after an 8 mL PBS/D<sub>2</sub>O buffer rinse. After the buffer rinse, the strength of the methyl bands increased roughly twofold. Based on adsorption studies by surface plasmon resonance, we hypothesize that the spectrum before rinsing is representative of a monolayer of d-13 DPPC with some surface coverage of vesicles and/or multilayers.<sup>4</sup> The effect of rinsing is to remove unfused vesicles or excess phospholipid, leaving a monolayer of phospholipid. Rinsing is not seen to significantly decrease or increase disorder in the phospholipid acyl chains.

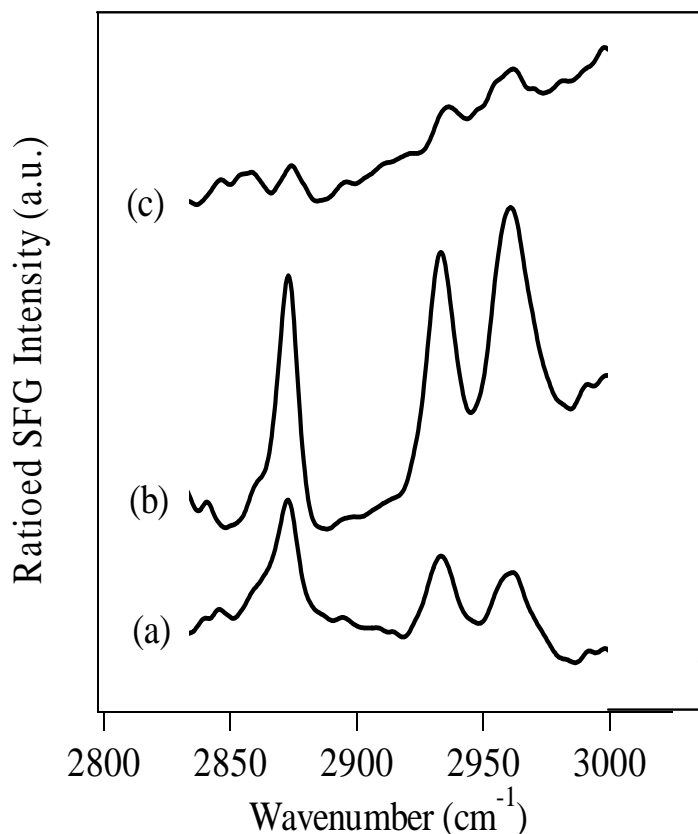


Figure 4: SFG spectra of an HBM before and after a buffer rinse and an air/ethanol rinse. The SFG spectrum in (a) is of the adsorbed d-13 DPPC in contact with the vesicle solution, (b) is taken after rinsing with 8 mL PBS/D<sub>2</sub>O buffer, and (c) is the result after air was pulled through the sample cell, followed by a 5 mL ethanol rinse, and refilled with PBS/D<sub>2</sub>O buffer.

The spectrum designated as (c) resulted after air was pulled through the sample cell, followed by a 5 mL ethanol rinse, and refilled with PBS/D<sub>2</sub>O buffer. Exposure of a phospholipid/alkanethiol HBM to air is thought to cause significant disruption of the phospholipid monolayer. Similarly, ethanol is a reasonable solvent for phospholipid and can remove the majority of the phospholipid monolayer. The very weak vibrational bands observed in spectrum (c) agree with the removal of most of the adsorbed d13- DPPC by the air/ethanol treatment. It is important to note that the appearance of strong methylene bands would indicate phospholipid monolayer disruption. However, the absence of strong methylene bands in conjunction with the disappearance of methyl bands implies instead removal of phospholipid from the surface.

The time frame of HBM formation observed by our SFG studies, roughly 45 minutes, is within the time frame of HBM formation observed by surface plasmon resonance.<sup>4</sup> SPR has demonstrated the phospholipid monolayers in HBMs are stable under continuous flow, but that unfused vesicles and/or multilayers are washed away after a buffer rinse. We observe an increase in the SFG band intensities after rinsing with buffer. This likely indicates that a significant amount of adsorbed phospholipid was not perturbed by the buffer washes, but perhaps unfused vesicles or multilayers (that could destructively interfere with the SFG signal) were removed. The observation that our SFG spectral features nearly disappear after an air and ethanol rinse indicates significant disruption and removal of the adsorbed phospholipid, in agreement with SPR results.

#### 4. CONCLUSION

In this paper, we present the first *in situ* monitoring of HBM formation via sum frequency generation. Due to our broad band approach, it is possible to follow the evolution of the phospholipid monolayer in real time. The SFG spectra indicate that the phospholipid layer is well-ordered with the acyl chains in an extended *all-trans* geometry. This study demonstrates that SFG can be used as an *in situ* spectroscopic probe of HBM formation and properties.

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